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Title

Enhanced angiogenesis by multiple release of platelet-rich plasma contents and basic fibroblast growth factor from gelatin hydrogels

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Abstract

The objective of this study is to evaluate angiogenic effects induced by biodegradable gelatin hydrogel granules incorporating mixed platelet-rich plasma (PRP) growth factor mixture (PGFM) and bFGF. The PRP was prepared by a double-spinning technique of animal bloods isolated, followed by the treatment with different concentrations of calcium chloride (CaCl_2) solution. The CaCl_2 solution treatment activated the platelets of PRP to allow releasing various growth factors, such as platelet-derived growth factor (PDGF)-BB, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)- β_1 , and epithelial growth factor (EGF). When determined in the PRP treated by different CaCl_2 solutions, the high amount of representative platelet growth factor, PDGF-BB, VEGF, EGF, and TGF- β_1 were detected in the CaCl_2 concentrations of 1, 2, and 4 wt% compared with higher or lower ones. The PRP treated was impregnated into gelatin hydrogels granules freeze-dried at 37 °C for 1 hr, and then the percentage of PGFM desorbed from the gelatin hydrogel granules was evaluated. The percent PDGF-BB, VEGF, EGF, and TGF- β_1 desorbed tended to decrease with the decreased CaCl_2 concentration. Taken together, the CaCl_2 concentration to activate PRP for PGFM release was fixed at 2 wt%. *In vitro* release tests demonstrated that the PGFM was released from the gelatin hydrogels granules with time. For the gelatin hydrogels incorporating PGFM and bFGF, the time profile of PDGF-BB or bFGF release was in good

correspondence with that of gelatin hydrogel degradation. The gelatin hydrogels granules incorporating mixed PGFM and bFGF were prepared and intramuscularly injected to a leg ischemia model of mice to evaluate the angiogenic effects in terms of histological and laser Doppler perfusion imaging (LDPI) examinations. As controls, the hydrogel granules incorporating bFGF, PGFM, and platelet-poor plasma (PPP) were used for the angiogenic evaluation. The number of blood vessels newly formed and the percentage of α -SMA-positive cells increased around ischemic sites injected with the gelatin hydrogel granules incorporating mixed PGFM and bFGF, in marked contrast to other control groups. The blood re-perfusion level of ischemic tissues was enhanced by the hydrogel granules incorporating mixed PGFM and bFGF, whereas no enhancement was observed for other groups. It is concluded that the dual release system of PGFM and bFGF from gelatin hydrogel granules is promising to enhance angiogenic effects.

Keywords

Gelatin hydrogel, PRP, bFGF, Controlled release, Angiogenesis

Introduction

Several treatments for therapeutic angiogenesis have been reported to demonstrate their potentials experimentally and clinically. Some clinical trials of gene therapy and bone marrow cells transplantation have been being carried out. On the other hand, the biosafety of genetic materials is not always confirmed [1-3]. In addition, the invasiveness to harvest bone marrow cells and the insufficient scientific knowledge and the related technology for bone marrow cells may be potential risks for cell therapy [4, 5]. Another choice is to make use of growth factor proteins, which enable cells to naturally induce their biological potentials for natural angiogenesis. If the growth factor can be used *in vivo* with the biological activity remaining, the growth factor-induced angiogenesis will be realistic.

Drug delivery system (DDS) is one of the practically possible technologies to positively modify the *in vivo* biological activity of growth factors. Biodegradable gelatin hydrogels have been designed and prepared for the controlled release of bioactive basic fibroblast growth factor (bFGF) [6], transforming growth factor (TGF)- β_1 [7], platelet-derived growth factor (PDGF)-BB [8], bone morphogenetic protein (BMP)-2 [9-11], and hepatocyte growth factor (HGF) [12, 13]. For example, bFGF could be released from the gelatin hydrogels to demonstrate the therapeutic potential of angiogenesis in the animal models of ischemic legs and hearts or the angiogenesis-induced enhancement

of therapeutic efficacy of cell transplantation of cells [14-21]. In addition, a clinical trial has been started to confirm the safety and feasibility of bFGF release from gelatin hydrogels for patients with clinical limb ischemia of intractable disease [22].

Platelet-rich plasma (PRP) is a source of autologous growth factors, including PDGF, vascular endothelial growth factor (VEGF), TGF- β_1 , and epithelial growth factor (EGF). Autologous PRP has been clinically used in a wide variety of surgical treatments for soft and hard tissues [23], such as the intractable wound [24], calvaria [25], or maxillofacial bone defects [26], cosmetic surgery [27, 28], spine literature [29], and neovascularization [30]. The platelet growth factors could be also released from the gelatin hydrogels to demonstrate their enhanced activity to induce angiogenesis, or the regeneration of knee meniscus [31, 32], intervertebral disc [33-35], and bone tissues [25, 36].

In this study, mixed PRP growth factor mixture (PGFM) and bFGF were incorporated into the gelatin hydrogels for the multiple releases and the angiogenic effect was evaluated comparing with the release of either PGFM or bFGF from the hydrogels. We have been reported that neovascularization can be induced with controlled release of bFGF from gelatin hydrogel. The therapeutic neovascularization generally involve both microvascular and macrovascular processes. At the microvasculature level, angiogenesis is defined as the sprouting and growth of capillaries, which, however, regress after cessation

of bFGF stimulation if pericytes are not recruited properly. Therefore, the stabilization of capillary networks newly created by mural cells (pericytes), known to be recruited by PDGF-BB, is essential for therapeutic neovascularization [37]. The process of capillaries maturation needs the recruitment of mural cells mediated by PDGF-BB [37, 38]. Taken together, in this study, we tried the multiple controlled release of PGFM and bFGF from the gelatin hydrogel to investigate the maturation of blood vessels newly formed in a murine model of hind limb ischemia. Following the intramuscular injection of gelatin hydrogel granules incorporating mixed PGFM and bFGF into a ischemia model animal. The *in vivo* angiogenesis was evaluated in terms of histological and laser Doppler perfusion imaging examinations. The CaCl_2 treatment condition of PRP to allow to release platelet growth factors was optimized based on the amount of growth factor released and the release pattern from the hydrogels granules. We examine the time profile of representative platelet growth factors from the hydrogel granules comparing with that of hydrogel degradation.

Materials and methods

Preparation of gelatin hydrogel granules

The gelatin sample of low endotoxin (beMatrix® Gelatin LS-H) with an isoelectric point (IEP) of 5.0, isolated by an alkaline process from the porcine skin, was kindly supplied from Nitta Gelatin Inc., Osaka, Japan. Biodegradable gelatin hydrogel granules were prepared by the glutaraldehyde (GA, Wako Pure Chemical Industries, Osaka, Japan) cross-linking of gelatin aqueous solution [6, 10, 11]. Briefly, after mixing 80 μ l of aqueous GA solution (25 wt%) with 40 g of aqueous gelatin solution (5 wt%) preheated at 40 °C, the mixed aqueous solution was cast into a polystyrene dish (127.8 \times 85.5 mm², Thermo Fisher Scientific Inc., MA, USA), followed by leaving for 12 hr at 4 °C to allow the chemical cross-linking of gelatin. The resulting hydrogel sheets were then placed in 100 mM glycine aqueous solution (1 ℓ), followed by agitation at room temperature for 1 hr to block the residual aldehyde groups of unreacted GA. The cross-linked hydrogel sheets were twice washed with double-distilled water (DDW). After washing, they were homogenized with Polytron PT-13000DM system (Central Scientific Commerce, Inc., Tokyo, Japan) and separated by sieves to obtain gelatin hydrogel granules at the diameter ranging from 32 to 52 μ m in the DDW-swollen state. Thereafter, the hydrogel granules were freeze-dried, and sterilized with ethylene oxide gas. The water content of gelatin hydrogel granules (the weight ratio of water present in

the hydrogel to the wet hydrogel) was 96 wt%, when calculated from the hydrogel weight before and after swelling in phosphate-buffered saline solution (PBS, pH 7.4) for 24 hr at 37 °C.

***In vitro* degradation assay**

To evaluate the pattern of hydrogel degradation, the gelatin hydrogel granules were agitated in two types of degradation solution (1 ml), which is PBS with or without 10 µg/ml of collagenase (collagenase 1A, Nitta Gelatin Inc., Osaka, Japan) at 37 °C. At different time intervals, the solution supernatant was removed and replaced with the same volume of fresh PBS solution with or without collagenase. The gelatin concentration in each supernatant was determined by optical density (OD) measurement at 260 nm by spectrometer (DU800, Beckman Coulter, CA, USA) to evaluate the time profile of hydrogel degradation. Experiment was performed independently for 3 samples at every sampling time.

Preparation of PRP and growth factors detection

BALB/c mice (8 weeks old; Shimizu Laboratory Animal Supply, Kyoto, Japan) were used. All animal experiments were approved by Kyoto University Committee for Animal Experimentation. Briefly, 15 mice were anesthetized by the intraperitoneal injection of pentobarbital (somnopenyl,

Kyoritsu Seiyaku, Tokyo, Japan) at a dose of 0.65 mg/kg body weights. PRP was prepared with a platelet-rich plasma kit (JP200; Japan Paramedic Co., Ltd, Tokyo, Japan) according to the method reported [39-41]. Briefly, animal bloods (10 ml) were collected into tubes containing acid-citrate-dextrose solution formula A (1:4 vol/vol) anticoagulant and centrifuged for 7 min at 450 ×G and 4 °C. Next, the yellow plasma with the buffy coat was carefully transferred into a BD Vacutainer® tube (Becton, Dickinson and Company, NJ, USA), and then centrifuged for 5 min at 1600 ×G and 4 °C. The platelet pellet was collected and the thrombolytic pellet in 1.0 ml of plasma was used as PRP while the supernatant was done for platelet-poor plasma (PPP). Before and after the PRP preparation, an aliquot was taken out to count the number of platelets. The density of platelets in the PRP prepared was increased by a factor of 5 when compared with that of the original blood.

To activate PRP for the growth factors release, the PRP prepared was mixed with CaCl_2 solution at concentrations of 0.4, 0.6, 0.8, 1, 2, 4, and 10 wt% at the vol/vol ratio of 7:1, followed by leaving for 1 hr at 37 °C. As controls, PRP was treated with mixed 2 wt% CaCl_2 and thrombin solution or the conventional freeze-and thaw method [42-44]. Briefly, PRP was frozen by liquid nitrogen for 1 min, and then rapidly thaw at 25 °C. The process was repeated 3 times.

The amount of growth factors, such as PDGF-BB, VEGF, EGF, and TGF- β_1 , released was

determined with Quantikine® enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, MN, USA), respectively. For TGF- β_1 ELISA, the samples were first treated with acid to lower the pH to 2.0 to denature latency-associated peptide to allow the detection of active TGF- β_1 . The supernatant was adjusted to the neutral pH before ELISA.

Evaluation of PDGF-BB desorption from gelatin hydrogel granules incorporating PGFM

To evaluate the extent of platelet growth factors impregnated into the gelatin hydrogel granules, the amount of growth factors desorbed from gelatin hydrogel granules incorporating PGFM with or without CaCl_2 treatment. The PRP was incubated with CaCl_2 solution at different Ca^{2+} concentration for 1 hr at 37 °C or treated with the freeze-and-thaw for 3 times. Then, 100 μl of treated- and non-treated-PRP was impregnated into 10 mg of freeze-dried gelatin hydrogel granules for 1 hr at 37 °C. The gelatin hydrogel granules incorporating PGFM were placed in 1 ml of PBS, and incubate for 3 hr at 37°C. The amount of growth factors in each PBS sample was determined by the ELISA similarly. The percent adsorption of growth factors to gelatin hydrogel granules was calculated by the following

formula.

Percent adsorption = $100 - (\text{the amount of desorbed growth factor} / \text{the amount of impregnated growth factor} \times 100)$.

The experiment was done independently three times for each granule.

Preparation of gelatin hydrogel granules incorporating mixed PRP treated and bFGF

An aqueous solution of human recombinant bFGF with an IEP of 9.6 (10 mg/ml) was kindly supplied by Kaken Pharmaceutical (Tokyo, Japan). The original bFGF solution was diluted with physiological saline solution (Otsuka pharmaceutical Co., Ltd. Tokyo, Japan) to give the solution concentration of 10 mg/ml. The aqueous solution containing 100 μg of bFGF (10 μl) and 100 μl of PGFM were dropped onto a freeze-dried gelatin hydrogel granule for impregnation of PGFM and bFGF into the granule. The bFGF and PGFM solutions were completely sorbed into the hydrogel granule by swelling at 37 °C for 1 hr, because the solution volume was less than theoretically required for the equilibrated swelling of hydrogels. Similarly, empty gelatin hydrogels without bFGF were prepared by adding PBS as the solution.

Release test of bFGF from gelatin hydrogel granules incorporating mixed PGFM and bFGF

To evaluate the bFGF release from the gelatin hydrogel granules incorporating mixed PGFM and bFGF, the gelatin hydrogels incorporating mixed PGFM and RI labeled-bFGF were incubated in two types of release solution (1 ml), which are PBS with and without 10 µg/ml of collagenase at 37 °C. After 6 hr incubation in PBS, there was a change from PBS to PBS with collagenase. At different time intervals, the solution supernatant was removed and replaced with the same volume of fresh PBS solution with or without collagenase. In this experiment, the PRP was treated with 2 wt% CaCl₂ solution for platelet activation, and bFGF was labeled with ¹²⁵I.

Basic FGF was radioiodinated with chloramine-T according to the method described by Greenwood *et al.* [45]. Briefly, 5 µl of Na¹²⁵I was added to 200 µl of 0.5 mg/ml bFGF solution in 0.5 M potassium PBS (pH 7.5) containing 0.5 M sodium chloride. Then 0.2 mg/ml of chloramine-T in 0.5 M potassium PBS (pH 7.5) containing 0.5 M sodium chloride (100 µl) was added to the solution mixture. After agitation at room temperature for 2 min, 100 µl solution containing 0.4 mg of sodium metabisulfate was added to the reaction solution to stop the radioiodination. The reaction mixture was passed through an anionic-exchange column to remove the uncoupled, free ¹²⁵I molecules from the ¹²⁵I-labeled bFGF.

The mixture of the aqueous solution containing 100 µg of ¹²⁵I labeled-bFGF (10 µl) and 100

μl of PGFM was impregnated into 10 mg of freeze-dried gelatin hydrogel granules for 1 hr at 37 °C. The supernatant was sampled at scheduled times and the amount of bFGF in each supernatant samples were determined by the γ-counter. The experiment was done independently three times for each granule.

Release test of PDGF-BB from gelatin hydrogel granules incorporating PGFM

To evaluate the platelet PDGF-BB release from the gelatin hydrogel granules incorporating PGFM, the gelatin hydrogels incorporating PGFM were incubated in two types of release solution (1 ml), which are PBS with and without 10 μg/ml of collagenase at 37 °C. After 12 hr incubation in PBS, there was a change from PBS to PBS with collagenase. At different time intervals, the solution supernatant was removed and replaced with the same volume of fresh PBS solution with or without collagenase. In this experiment, the PRP was treated with 2 wt% CaCl₂ solution for platelet activation, and 100 μl of treated PRP was impregnated into 10 mg of freeze-dried gelatin hydrogel granules for 1 hr at 37 °C. The supernatant was sampled at scheduled times and the amount of PDGF-BB in each supernatant samples were determined by the ELISA similarly. The experiment was done independently three times for each granule.

***In vivo* experiments**

Hind limb ischemia was created in 8-week-old male BALB/c mice. Briefly, after the mice were anesthetized by an intraperitoneal pentobarbital (0.65 mg/kg) injection, the right groin area was shaved. The entire right saphenous artery and vein, right external iliac artery and vein, and deep femoral and circumflex arteries and vein were ligated, cut, and excised to obtain a murine model of severe hind limb ischemia. The BALB/c mice treated were randomly classified to 6 groups (n=10 per groups): hydrogel granules incorporating mixed PGFM and bFGF (PGFM + bFGF), gelatin hydrogel granules incorporating either PGFM or bFGF (PGFM or bFGF), gelatin hydrogel granules incorporating PPP (PPP), no-treatment (NT), and PGFM- and bFGF-free PBS (PBS). All drugs were homogeneously injected into 5 sites in the thigh muscle of mouse ischemic hind limb by using a 27-gauge needle.

The animals were re-anesthetized by an intraperitoneal injection of pentobarbital (0.65 mg/kg) 1 wk after treatment, and the following measurements were performed to evaluate the *in vivo* angiogenesis. Each experiment was done three times independently otherwise mentioned.

Hind limb Blood perfusion

Hind limb blood perfusion was assessed by using a laser Doppler blood perfusion image

(LDPI) analyzer (moorLDI™, Moor Instruments, Deven, UK) 1 wk after treatment [14]. To evaluate the influence of surgical procedure on the blood flow, the blood perfusion of normal hind limb and treated one were measured with LDPI. To minimize external influence, such as light and temperature, the perfusion result was expressed as the LDPI index which is the ratio of the blood perfusion in the right (ischemic) limb to that in the left (non-ischemic) limb of the same mouse [14, 18, 46]. The blood flow was measured in the site of vascular resection.

Histological examination

The specimens of muscular tissues were fixed with 4 wt% paraformaldehyde (PFA) in 0.1 M phosphate-buffered solution for overnight at 4 °C and decalcified with Plank–Rychlo’s solution overnight at room temperature. After decalcification, the samples were embedded in paraffin. Each specimen was cut into slices of the thigh, 4 µm thick of cross section. For histological analysis, the section was stained with hematoxylin and eosin (HE) dyes, the number of blood vessels newly formed in the thigh was counted in a blind fashion. The number ratio of blood vessels newly formed in the right (ischemic) limb that in the left (non-ischemic) limb of the same mouse was calculated.

Immunohistological analysis

The animals were re-anesthetized by an intraperitoneal injection of pentobarbital (0.65 mg/kg) 1 week after treatment, and the mice were perfusion-fixed with 4 wt% PFA solution. The ischemic thigh was decalcified and embedded in paraffin. Paraffin sections (5 μ m thickness) of tissues were stained with a mouse monoclonal anti- α -smooth muscle actin antibody (α -SMA; Sigma-Aldrich Japan K.K., Tokyo, Japan). From each mouse, all fields on tissue sections were photographed with a digital camera (Olympus, Tokyo, Japan). The number of α -SMA (vascular smooth muscle marker)-positive vessels was counted in a blind fashion. The number of blood vessels was counted for HE staining section. The percentage of number of α -SMA-positive blood vessels to that of total blood vessels was calculated. Each experiment was done three times independently.

Statistical analysis

All the statistical data were expressed as the mean \pm standard error of the mean. The data were analyzed by ANOVA to determine the statistical significance between the two mean values. The p value less than 0.05 were considered to be significant.

Results

CaCl₂ treatment of PRP for the release of platelet growth factors

Table 1 shows the concentration of various growth factors released from PRP after the freeze-and-thaw treatment. The treatment activated the platelet of PRP to release various growth factors. Based on the profile of growth factors released, PDGF-BB, VEGF, EGF, and TGF- β_1 were selected as the representative platelet growth factor because of the highest amount and the activity to enhance the maturation of blood vessels newly formed [38, 47-53].

Figure 1 shows the concentration of PDGF-BB released from PRP after the CaCl₂ treatment. Upon treating with 0.8, 1, 2, and 4 wt% of CaCl₂ solution, the concentration of PDGF-BB in the soluble fraction of PRP was significantly high compared with that of non-treated PRP. On the other hand, the treatment of 10 wt% CaCl₂ solution did not increase the PDGF-BB amount. The concentration of EGF in the soluble fraction of PRP was significantly high compared with the non-treated PRP and the 10 wt% CaCl₂ solution treated PRP (Figure 2A). The amount of VEGF extracted from PRP was higher in PRP treated with CaCl₂ compared with that of non-treatment (Figure 2B). In this study, PDGF was noted because it has on ability to enhance the maturation of blood vessels. In addition, PDGF is one of the main

factors present in PRP. On the contrary, the amount of TGF- β_1 in PRP treated with 1, 2, and 4 wt% CaCl₂ was higher than that of 10 wt% CaCl₂ treated PRP, although it was not significant (Figure 2C).

Adsorption of growth factors to gelatin hydrogel granules incorporating PGFM and the *in vitro* profiles of PDGF-BB release

Figure 3 shows the adsorption patterns of growth factors to gelatin hydrogel granules incorporating PGFM. For PRP treated with 0.8, 1, 2, and 4 wt% CaCl₂ for 1 hr at 37 °C, the amount of PDGF-BB adsorption was significantly low compared with that treated with 0.4 and 0.6 wt% CaCl₂ (Figure 3A). The pattern of VEGF, EGF and TGF- β_1 adsorption from the hydrogel granules incorporating PGFM was influenced by the CaCl₂ concentration (Figures 3 B, C, and D). On the other hand, Figure 4 shows the adsorption patterns of growth factors to gelatin hydrogel granules incorporating mixed PGFM and bFGF and time courses of bFGF from gelatin hydrogel granules incorporating mixed PGFM and bFGF in PBS with or without collagenase. When mixed PGFM and bFGF were impregnated to gelatin hydrogel granules, the adsorption rate of bFGF to gelatin hydrogel granules was about 50 % of impregnated bFGF (Figure 4A). In the release in PBS, about 60 % of bFGF was released from the hydrogel granules. When

mixed PGFM and bFGF were impregnated to gelatin hydrogel granules, the initial burst of bFGF was increased compared with that of single impregnation of bFGF. However, when the release test was performed in PBS containing collagenase, the bFGF was release with time (Figure 4B).

Figure 5 shows the time courses of PDGF-BB from gelatin hydrogel granules incorporating PRP treated with 2 wt% CaCl_2 solution in PBS with or without collagenase. In the release in PBS, PDGF-BB was not released from the hydrogel granules, although the initial burst of small amount in release was observed. However, when the release test was performed in PBS containing collagenase, the PDGF-BB was release with time. The similar addition effect of collagenase on the time profile of hydrogel degradation was seen. A good correlation of time profiles was observed between the PDGF-BB release and the hydrogel degradation.

Angiogenic effects of gelatin hydrogel granules incorporating mixed PGFM and bFGF

Figure 6 shows the *in vivo* angiogenic effects of gelatin hydrogel granules incorporating PGFM with or without bFGF combination 1 week after implantation. The PRP was treated with 2 wt% CaCl_2 solution. From the histological sections, the new formation of blood vessels was observed for the hydrogel granules incorporating mixed PGFM and bFGF. The number of blood vessels newly formed

was significantly high compared with that of other groups. The vessels number was not increased by injection of hydrogel granules incorporating PGFM, bFGF or PBS. PBS solution containing PGFM, bFGF or mixed PGFM and bFGF did not increase the number of new blood vessels (data not shown). The percentage of α -SMA-positive blood vessels increased by the granules incorporating mixed PGFM and bFGF to a significantly high extent compared with that of other controls. No difference in the vessels number between the right and left limbs was observed for the non-treated and empty granules injected groups. This indicates that neither the surgical procedure nor the granules injection induced angiogenesis.

Figure 7 shows blood perfusion levels in the ischemic limb after the injection of different gelatin hydrogel granules incorporating mixed PGFM and bFGF. The injection of hydrogel granules incorporating mixed PGFM and bFGF significantly enhanced the perfusion index, in remarked contrast to that of other groups.

Discussion

The present study demonstrates that the multiple releases of PRP growth factors and bFGF from gelatin hydrogel granules was effective in increasing the number of blood vessels newly formed and the blood flow level in the ischemic legs. In addition, the number of α -SMA-positive blood vessels was also increased, which suggests the maturation enhancement of blood vessels.

In the living tissue, it is well known that various growth factors interact with the components of extracellular matrix, such as acidic polysaccharides and collagen, through various intermolecular interaction forces [54]. In addition, the physicochemical interactions serve to maintain and enhance the *in vivo* biological function of growth factors. For instance, bFGF with an IEP of 9.6 forms polyion complexes with heparin and heparan sulfate *in vivo*, resulting in the stabilization and regulation of biological activities [43, 55]. The manner of natural physicochemical interactions is a good example to design the controlled release system of growth factors. Based on this, we have developed the biodegradable hydrogels of gelatin [9-11, 56]. The physicochemical property of gelatin, such as the electric nature, can be readily modified by the preparation procedure. For example, gelatin prepared with an alkaline process from collagen has an IEP of 5.0 and is negatively charged at the physiological pH, whereas an acidic process of collagen gives a positively charged gelatin with an IEP of 9.0. This electric

nature of gelatin can be used to interact electrostatically with growth factors of different IEPs [56].

Recently, human recombinant growth factors have become commercially available and have been experimentally or clinically used for tissue regeneration applications. However, only when the growth factor in the solution form is used *in vivo*, the biological activity is not always expected. This is because it is un-stable *in vivo* due to the rapid enzymatic digestion or deactivation. Therefore, their delivery technology is highly required to enable the growth factor to efficiently enhance the biological functions.

We have succeeded in the controlled release of various growth factors as well as PRP from the gelatin hydrogels [6-13]. It has been recognized that when activated, the platelets of PRP release various growth factors through the degranulation of α granules and the factors released exhibit various biological activities [23]. For example, collagen and thrombin trigger the platelets aggregation and the subsequent release of platelet growth factors [57]. It is experimentally confirmed that the gelatin of a denatured collagen can function to trigger the platelet activation for growth factor release [36]. When impregnated into the hydrogel freeze-dried, it is likely that the PRP is activated by the contact with gelatin molecules to release the growth factors. The factors released interact with gelatin molecules during the impregnation procedure. Consequently, the PRP growth factors can be released from the gelatin hydrogels as a result of the hydrogel degradation. However, the contact of PRP with gelatin molecules is not always strong

enough to release out the PRP growth factors. In this study, the treatment of PRP with CaCl_2 solution was tried to accelerate the release of PRP growth factors. The CaCl_2 treatment is one of the well-known methods to activate PRP [41, 58].

The adsorption experiment demonstrated that the percent of PRP growth factors desorbed increased with decrease in the CaCl_2 concentration. This can be explained in terms of solution ionic strength. It is recognized that the growth factor physicochemically interacts with the gelatin molecules of hydrogels while one of the main interactions is the electrostatic. It is, therefore, likely that the increased CaCl_2 concentration suppresses the interaction between the PRP growth factor and gelatin molecules, resulting in increased the desorption percentage. In addition, the pattern of PDGF-BB release from the hydrogel granules incorporating PGFM is influenced by the CaCl_2 concentration. When the concentration was 4 wt%, higher amount of PDGF-BB release for the initial time period was observed compared with that of granules incorporating PGFM treated with 2 wt% CaCl_2 solution. Taken together, the CaCl_2 concentration for PRP treatment was fixed at 2 wt%. The time profile of PDGF-BB release from the granules incorporating 2 wt% CaCl_2 treated-PRP was in a good accordance with that of granules degradation. This indicates that the growth factor release is based on the degradation of release carrier hydrogels. When PRP treated with the freeze-and-thaw method, the concentration of PDGF-BB in the

soluble fraction of PRP was significantly high compared with that of CaCl_2 -treated PRP. However, the activation of PRP by the freeze-and-thaw method is not always suitable because of the complicated process in clinic.

The CaCl_2 treatment affects the amount of platelet growth factor released and the adsorption profile to the hydrogel granules incorporating PGFM (Figures 1 and 2). In this study, PDGF-BB, VEGF, EGF, and $\text{TGF-}\beta_1$ were selected as the representative platelet growth factor because of the high amount and the activity to enhance the maturation of blood vessels newly formed [47-49, 51, 53, 59]. In the CaCl_2 concentration of 1, 2, and 4 wt%, the amount of growth factors was higher than that of higher or lower CaCl_2 concentrations. The treatment at the lower concentration would be not enough to activate the platelet. On the other hand, it is possible that the strong activation of platelet with CaCl_2 solution of higher concentration results in the release out of platelet ingredients. The ingredients may suppress the activity of platelet growth factor.

We have demonstrated successful angiogenic effects on ischemic legs and hearts by biodegradable acidic gelatin hydrogels for the controlled release of bFGF [10, 11]. The bFGF release from the gelatin hydrogels was therapeutically effective in various animal models (i.e., either non-diabetic or diabetic) for acute myocardial infarction, prevascularization for cardiomyocyte transplantation to the

ischemic heart, limb ischemia, and bone regeneration of sternum [14–21]. In the release system, both the biodegradable gelatin hydrogels of release carrier and the bFGF of an angiogenic factor are clinically available at present. Based on the results of animal experiments, several clinical trials have been started to confirm the safety and feasibility of bFGF release from the gelatin hydrogel in patients with clinical limb ischemia. The gelatin hydrogel for bFGF release is simple, safe, and effective from the clinical viewpoint. It is not necessary to use genetic materials and collect cells to be transplanted. In addition, no elevation of bFGF serum level was reported in the clinical case [22]. However, the bFGF release system has some rooms to be improved for the clinical applications. It is practically necessary for the treatment of patients with severe conditions to enhance the number of blood vessels newly formed and their biological maturation. The enhancement of blood vessels maturation is one of the practical strategies to treat such patients in the severe condition. There are several researches on the maturation of blood vessels in terms of growth factors usage. PRP is a source of autologous growth factors, including PDGF-BB, VEGF, EGF, and TGF- β . Several growth factors become intricately involved in angiogenesis. The PDGF-BB, VEGF, and TGF- β play an important role as proangiogenic stimulators. Several studies reported that PDGF-BB has a potent arteriogenic effect that promotes differentiation of endothelial cells [60], and VEGF is known to stimulate angiogenesis after ischemia [61], and TGF- β promote cell mitosis [53]. However, other

studies indicated that several growth factors, such as PDGF-BB and TGF- β , inhibit the angiogenic effect of bFGF [62-65]. These studies were evaluating the angiogenic effect using mixed solution of growth factors. Moreover, the amounts of growth factors was used transiently at high concentration. On the other hand, we used gelatin hydrogel granules incorporating PGFM and bFGF in this study, this gelatin hydrogel granules was degraded for 2 weeks, and sustained release the encapsulated growth factors. Thus, our sustained-release system and previous reports are fundamentally different. Among them, PDGF-BB is one of the powerful growth factors to allow the blood vessels to mature functionally [38, 49]. The finding gives us the idea that the multiple releases of PGFM and bFGF will enhance the maturation of blood vessels. Mooney *et al.* reported that the dual release of VEGF and PDGF enhanced the maturation of blood vessels newly formed compared with that of single VEGF release. The *in vivo* experiments clearly indicate that the dual release of PRP treated and bFGF from the biodegradable gelatin hydrogel granules promoted not only angiogenesis in a mouse model of limb ischemia, but also the maturation of blood vessels whereas the release of either PGFM or bFGF was not effective. There have been reported on the synergistic effects of different drugs on the enhancement of tissue regeneration [66-68] and tissue maturation. The dual release technology is available for any type of drug and to promote the regeneration of various tissues.

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Figure legend

Figure 1.

Concentration of PDGF-BB released from PRP after the treatment of CaCl_2 solution at different Ca^{2+} concentrations, the mixed solution of 2 wt% CaCl_2 and thrombin, and freeze-and-thaw treated. *, $p<0.05$; significant different from the values of non-treated PRP. †, $p<0.05$; significant different from the values of 0.4 wt% CaCl_2 -treated PRP. ‡, $p<0.05$; significant different from the values of 10 wt% CaCl_2 -treated PRP.

Figure 2.

Concentration of growth factors released from PRP after the treatment of CaCl_2 solution at different Ca^{2+} concentrations, the mixed solution of 2 wt% CaCl_2 and thrombin, and freeze-and-thaw treated. (A) Concentration of EGF released from PRP. *, $p<0.05$; significant different from the values of non-treated PRP. †, $p<0.05$; significant different from the values of 10 wt% CaCl_2 -treated PRP. ‡, $p<0.05$; significant different from the values of freeze-and-thaw treated PRP. (B) Concentration of VEGF released from PRP. *, $p<0.05$; significant different from the values of freeze-and-thaw treated PRP. (C) Concentration of $\text{TGF-}\beta_1$ released from PRP.

Figure 3.

Adsorption of growth factors to gelatin hydrogel granules. After treatment of CaCl_2 solution at different Ca^{2+} concentrations and the mixed solution of 2 wt% CaCl_2 and thrombin, the PRP treated was incorporated into the gelatin hydrogels at 37 °C for 1 hr. The concentration of growth factors desorbed from gelatin hydrogel granules incorporating PGFM treated was detected by ELISA. (A) The percent adsorption of PDGF-BB to gelatin hydrogel. *, $p < 0.05$; significant different from the values of 0.4 wt% CaCl_2 treated PRP. †, $p < 0.05$; significant different from the values of 0.6 wt% CaCl_2 treated PRP. ‡, $p < 0.05$; significant different from the values of the mixed solution of 2 wt% CaCl_2 and thrombin. (B) The percent adsorption of VEGF to gelatin hydrogel. (C) The percent adsorption of EGF to gelatin hydrogel. (D) The percent adsorption of $\text{TGF-}\beta_1$ to gelatin hydrogel.

Figure 4.

Adsorption of bFGF to gelatin hydrogel granules and time course of bFGF release from gelatin hydrogel granules incorporating mixed PGFM and ^{125}I -labeled bFGF. (A) The percent adsorption of bFGF to gelatin hydrogel. (B) The time course of bFGF release from gelatin hydrogel granules incorporating

mixed PGFM and ^{125}I -labeled bFGF (○) and gelatin hydrogel granules incorporating ^{125}I -labeled bFGF (●) in PBS with or without collagenase. The PBS solution was changed to that with collagenase at the time indicated with arrow.

Figure 5.

The time course of PDGF-BB release from gelatin hydrogel granules incorporating PRP treated with 2 wt% CaCl_2 solution (○) and hydrogel degradation (●) in PBS with or without collagenase. The PBS solution was changed to that with collagenase at the time indicated with arrow.

Figure 6.

In vivo angiogenic effects of gelatin hydrogel granules incorporating PRP with or without bFGF 1 week after implantation. (A) Histological section ((a-f) hematoxylin and eosin and (g-l) anti- α -SMA immunohistochemical staining) of tissues injected with different gelatin hydrogel granules: (a, g) hydrogels incorporating PGFM and bFGF, (b, h) PGFM, (c, i) bFGF, (d, j) PPP or (e, k) PBS, and (f, l) non-treatment. The scale bar indicates 100 μm . (B) The number ratio of blood vessels newly formed around tissues injected with gelatin hydrogel granules. The number ratio of blood vessels newly formed

in the right (ischemic) limb was calculated as for that in the left (non-ischemic) limb of the same mouse.

Ratio of 1.0 was calculated in non-treated mice. *, $p < 0.05$; significant different from the value of gelatin

hydrogel granules incorporating PPP. †, $p < 0.01$; significant different from the value of gelatin hydrogel

granules incorporating PBS. ‡, $p < 0.01$; significant different from the value of non-treatment. (C)

Percentage of anti- α -SMA antibody positive blood vessels around the tissue injected with gelatin

hydrogel granules. The percentage of number of α -SMA-positive blood vessels to that of total blood

vessels was calculated. *, $p < 0.05$; significant different from the value of gelatin hydrogel granules

incorporating PGFM. †, $p < 0.05$; significant different from the value of gelatin hydrogel granules

incorporating bFGF.

Figure 7.

In vivo blood perfusion effects of gelatin hydrogel granules incorporating PGFM with or without bFGF 1

week after implantation. (A) Dorsal view of mouse image. The blood flow was measured in boxed area.

(B) The image of laser Doppler perfusion of tissues injected with different gelatin hydrogel granules. (a)

Hydrogels incorporating PGFM and bFGF, (b) PGFM, (c, i) bFGF, (d) PPP or (e) PBS, and (f)

non-treatment. (C) The percentage of blood perfusion in the newly formed blood vessels around the tissue

injected with gelatin hydrogel granules. The percentage of blood perfusion in the right (ischemic) limb was calculated as for that in the left (non-ischemic) limb of the same mouse. The baseline is perfusion index of non-ischemic limb.

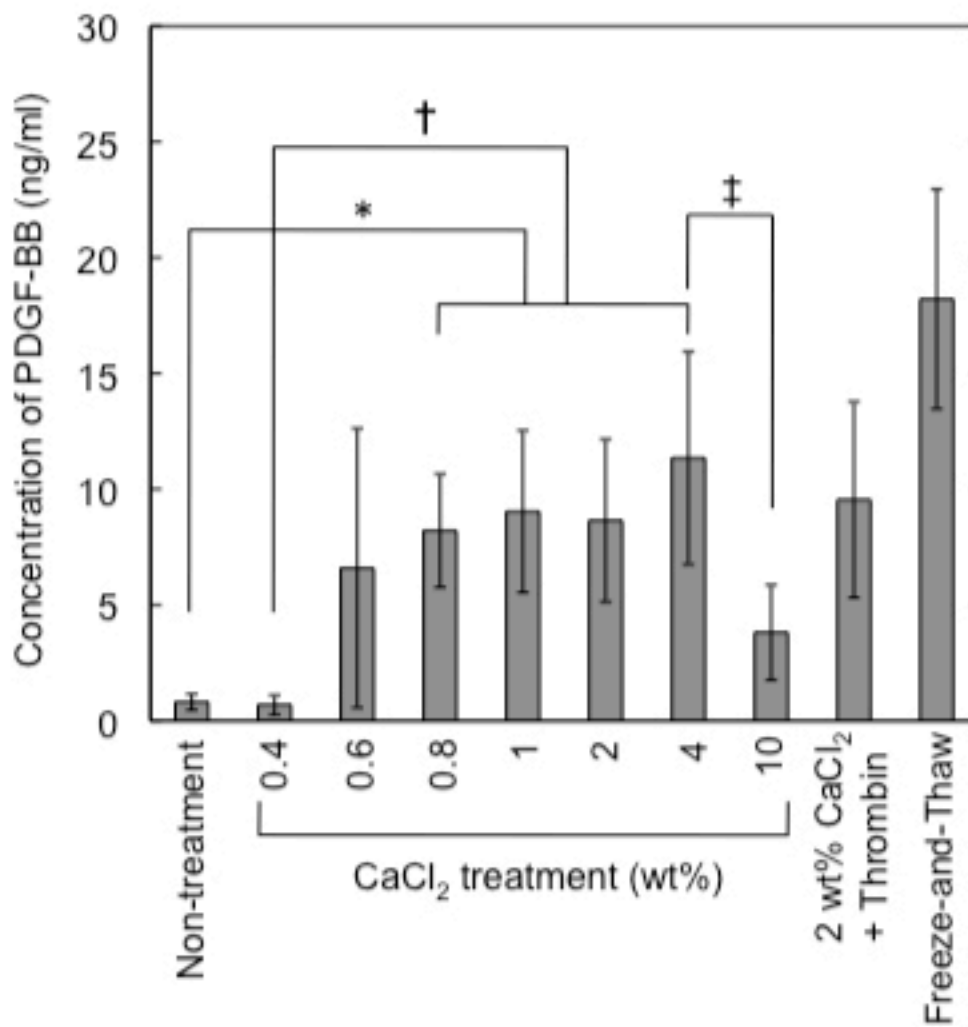
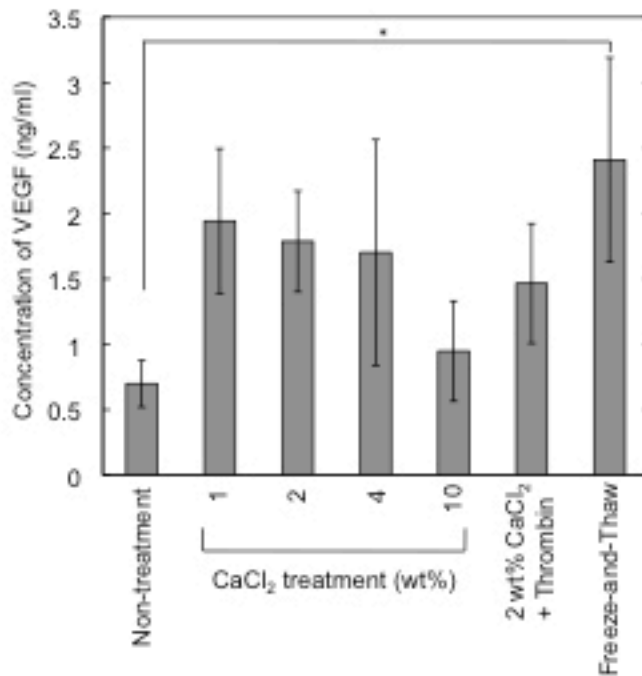
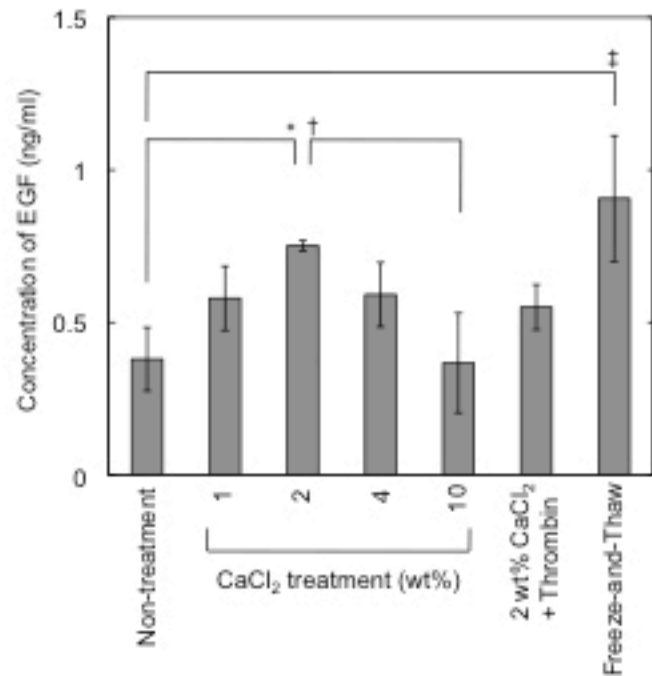


Figure 2

(A)



(B)



(C)

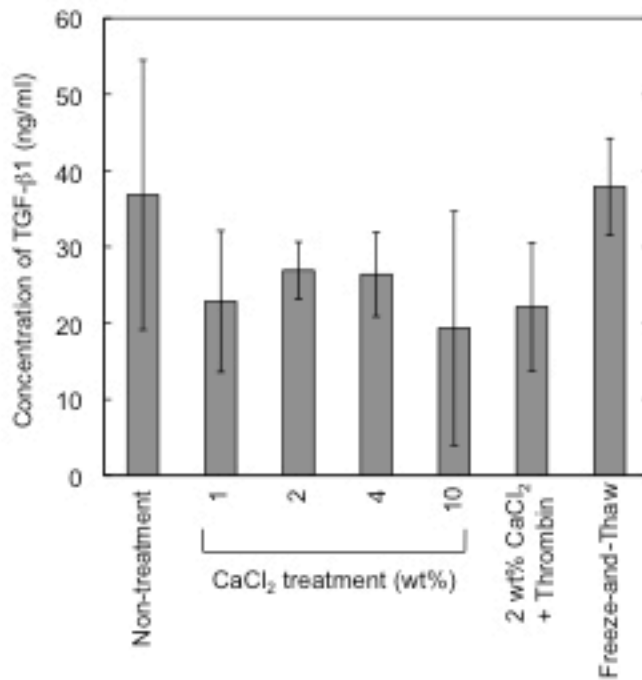


Figure 3

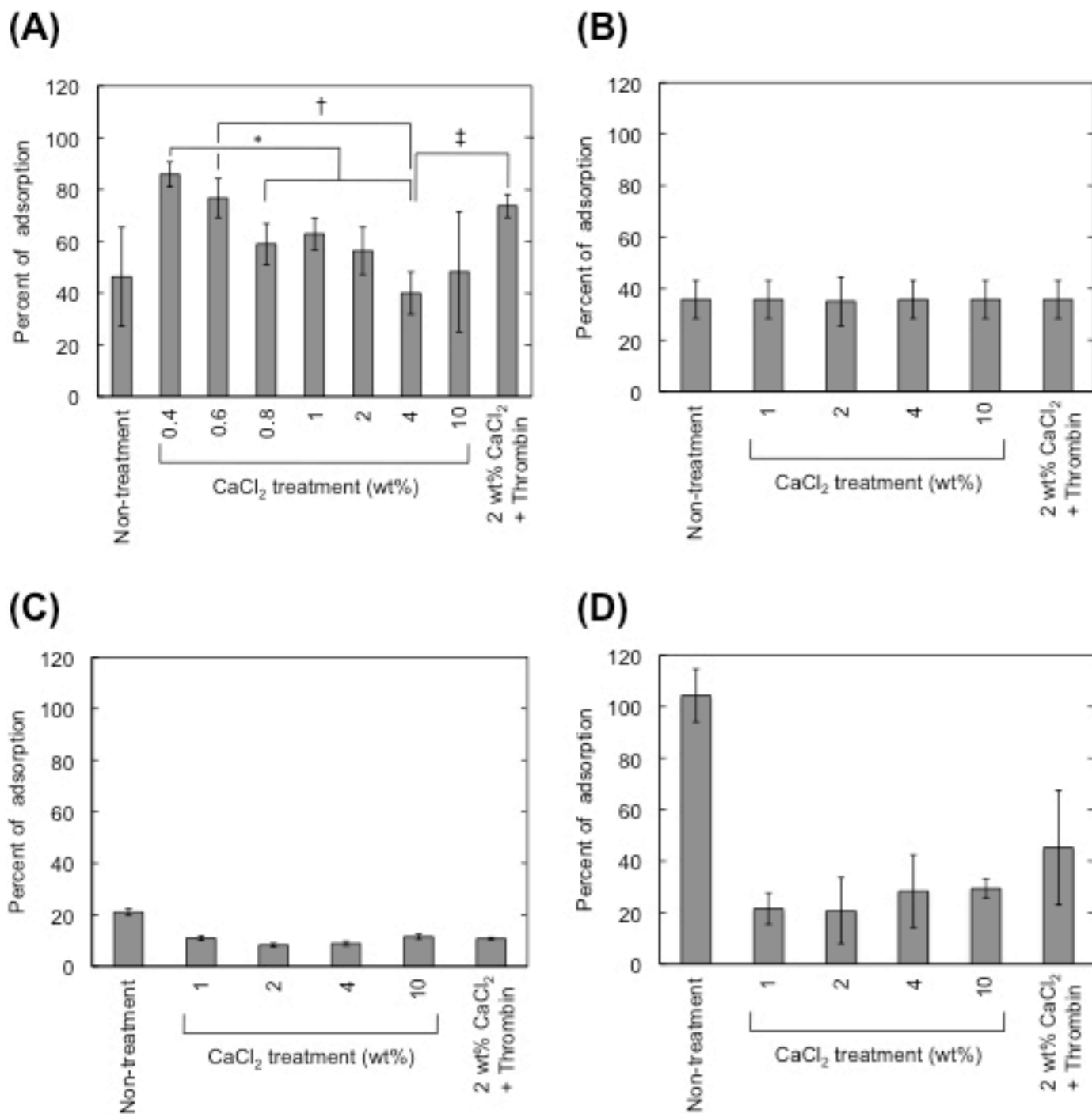


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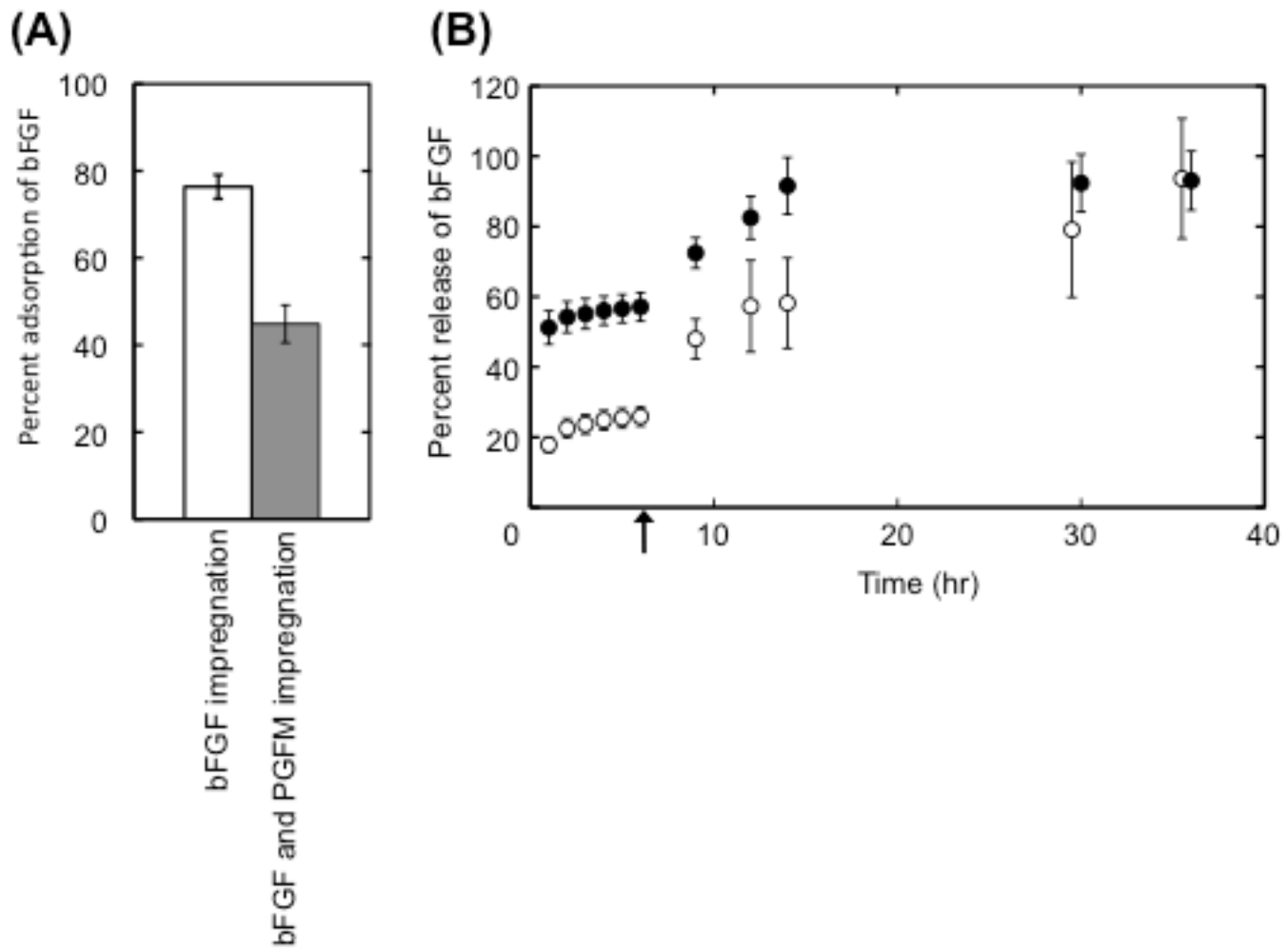


Figure 5

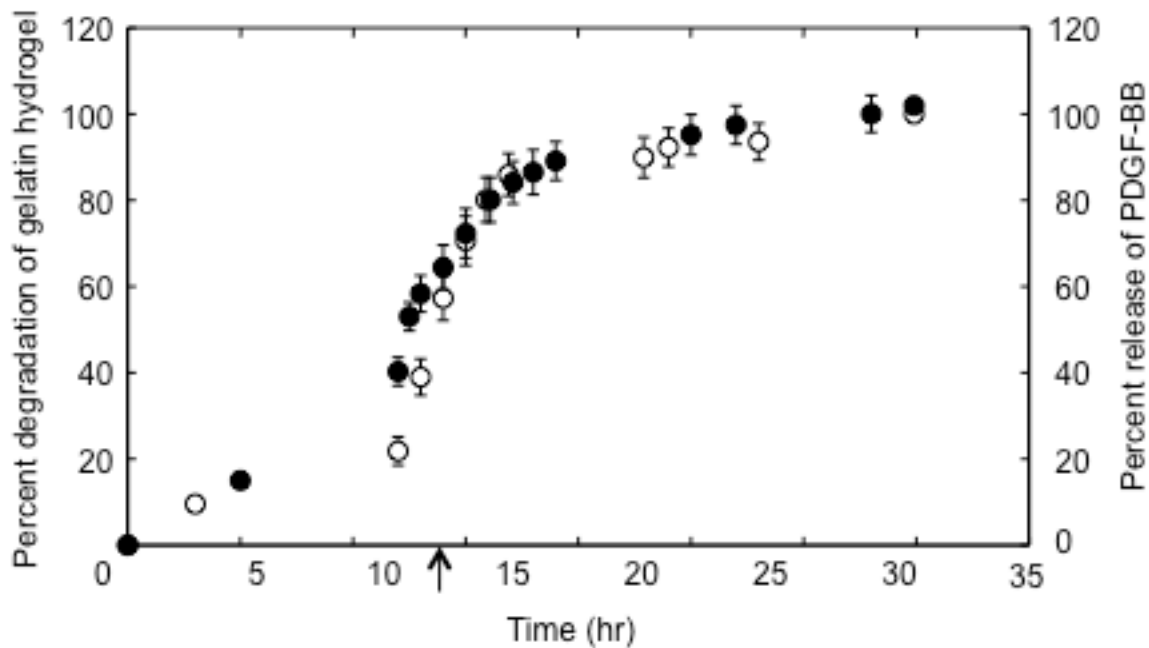


Figure 6

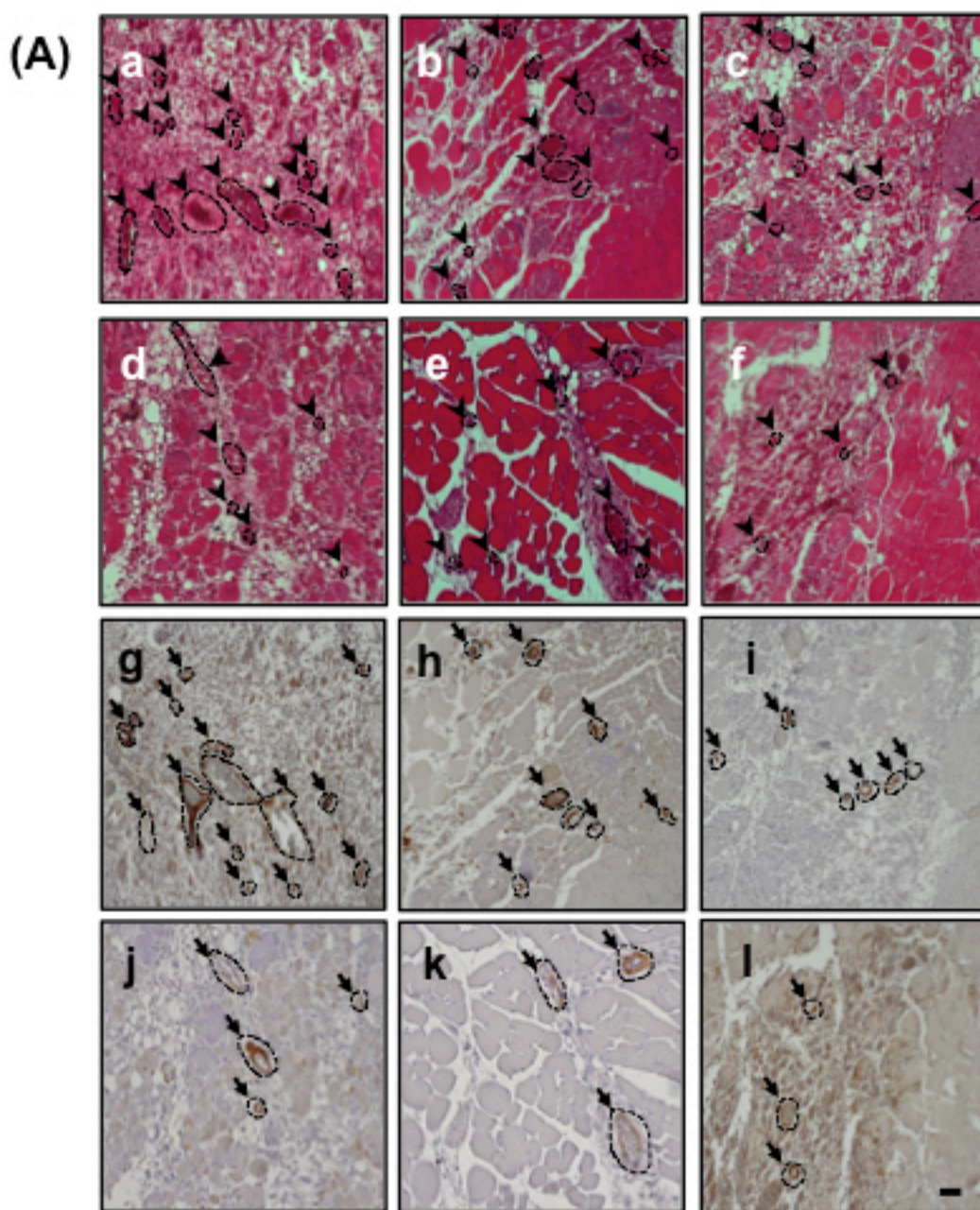


Figure 6

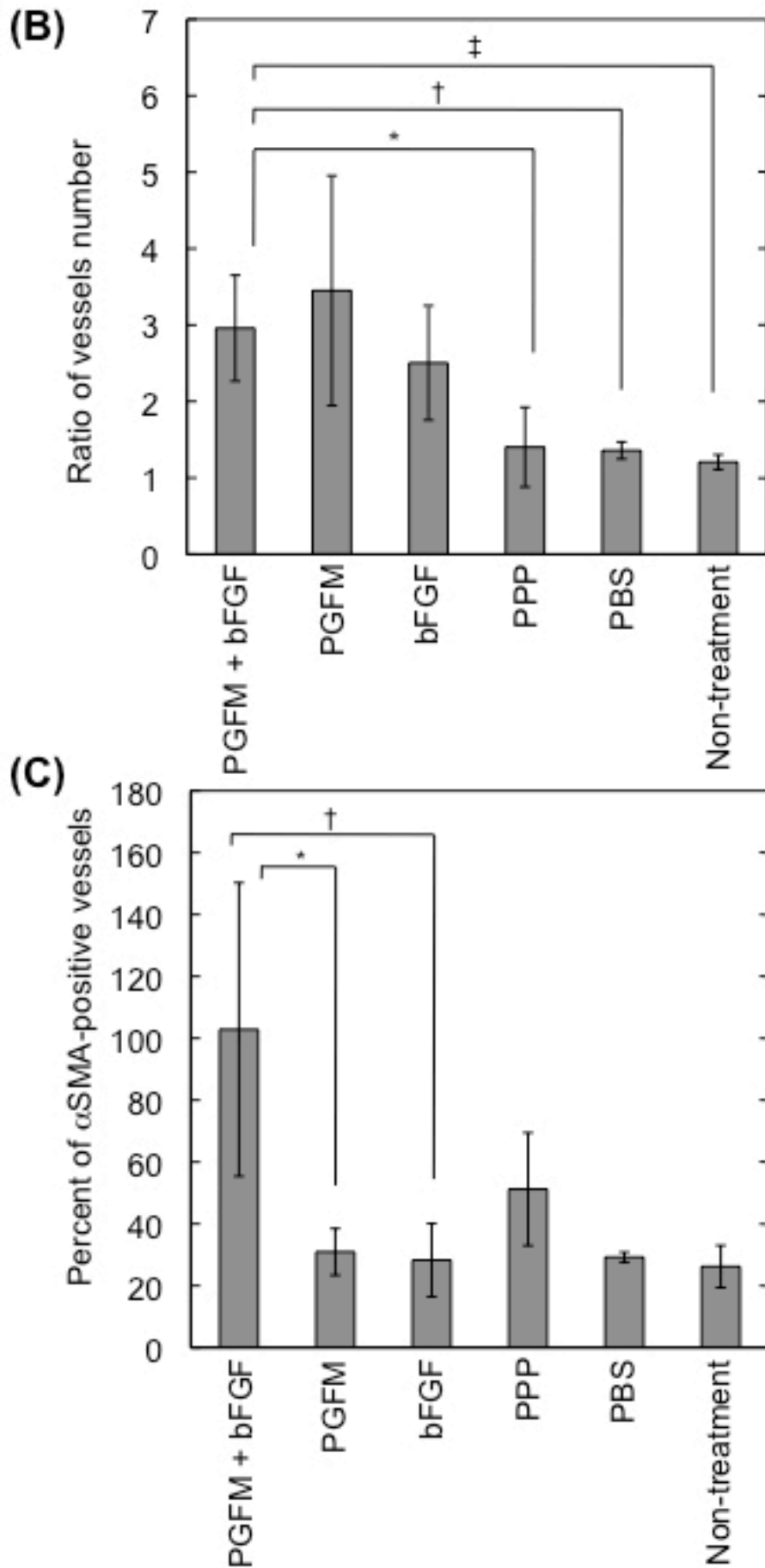


Figure 7

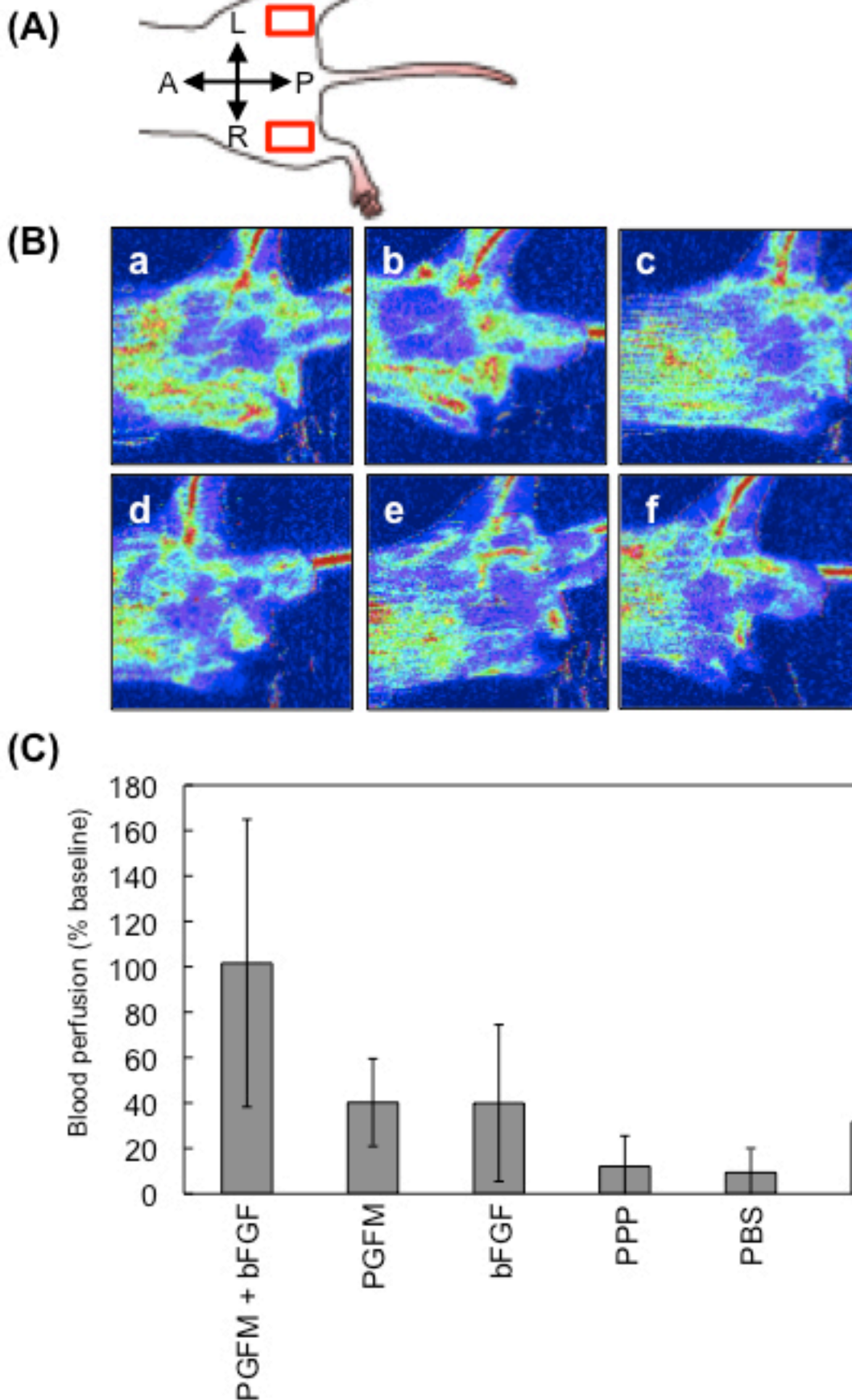


Table 1 Concentration of growth factors in PRP

Growth factor	Concentration (pg/ml)
PDGF-BB	9440 ± 1620
VEGF	2040 ± 971
TGF- β_1	30500 ± 20500
EGF	906 ± 206
bFGF	32.6 ± 8.7

The growth factors were extracted from PRP through the freeze-and thaw treatment.